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13. ABSTRACT ***(Maximum 200 Words)***

Radiographic mammary microcalcifications are one of the most pertinent diagnostic markers of breast cancer. Breast tissue calcification in the form of hydroxyapatite (HA) crystals is strongly associated with malignant disease. HA crystals play a well-described role in a number of disease processes unrelated to the breast, including severe cartilage destruction. The characteristics of HA believed to be essential to pathogenesis include their ability to induce 1) cell replication and 2) matrix metalloprotease (MMP) production, both of which may be relevant to growth and/or metastasis of breast cancer. The goal of this project is to test the hypothesis that HA crystals facilitate breast cancer progression because of their ability to induce mitogenesis and MMP production. We have shown that calcium HA crystals enhance mitogenesis in normal HF, non-malignant mammary cell lines (HMEC and Hs578BSt) and breast cancer cell lines (MCF-7 and Hs578T) alone or in co-culture. HA crystals enhance the production of a variety of MMP in normal or breast cancer cell lines alone or in co-culture. In addition, HA crystals can enhance PGE_2 production in some mammary cell lines by upregulation of cyclo-oxygenases (COX) 1 and 2. Finally, HA crystals induce IL-1b but not TNF-a in HF. Taken together, these data support the active role of calcium HA in breast cancer progression.

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Title: Biological effects of calcium hydroxyapatite crystals in breast cancer.
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Introduction

Radiographic mammary microcalcifications constitute one of the most pertinent markers of both benign and malignant lesions of the breast. Analysis of these microcalcifications by electron microscopy, microprobe analysis and X-ray diffraction has shown that in breast tissue two principle types of calcifications can be distinguished according to their structure and chemical composition (Harris, Morrow et al. 1993). Type I microcalcifications are composed of calcium oxalate in the form of weddellite crystals and type II microcalcifications consist of calcium phosphates in the crystalline form of hydroxyapatite (HA), ($Ca_{10}(PO_4)_5(OH)_2$), which is also the basic calcium phosphate found in mature bones and teeth. There is evidence that calcium phosphate and oxalate tend to be associated with different kinds of breast lesions (Busing, Keppler et al. 1981). The presence of oxalate-type microcalcification appears to be a reliable criterion in favour of the benign nature of the lesion or, at most, of an *in situ* lobular carcinoma and are rarely associated with malignancy (Radi 1989; Going, Anderson et al. 1990). In contrast, the calcifications associated with malignant breast lesions are generally formed by hydroxyapatite (Frappart, Boudeulle et al. 1984).

Although their diagnostic value is of great importance radiographically, the genesis of breast calcifications is unclear. The occurrence of microcalcifications has not been shown to be significantly associated with age or primary tumour size. However, several studies have shown that survival of patients with mammographic microcalcification was significantly shorter than those without (Tsuchiya, Kanno et al. 1996),(Holme, Reis et al. 1993). A recent study by Tabar *et al.* also showed that the relative hazard of death from breast cancer was five times higher for tumours with casting-type calcifications than that for circular lesions with no calcifications (Tabar, Chen et al. 2000). There have been numerous histological ultrastructure studies of HA deposits in breast carcinomas. However, despite their potent biological effects in other systems and their association with poorer survival in breast cancer patients, to date there have been no investigations of their potential role in the growth and progression of breast tumours. In the present study, we investigated the pathogenic potential of calcium HA crystals in human breast cancer cell lines and human fibroblasts by studying their ability to induce mitogenesis, and upregulate PGE_2 and MMP production.

This annual report covers research for the period August 1st, 1999 to July 31st, 2000. Although this should be the final report, an extension of the award period has been sought. This extension is due to the principal investigators change of institutions in July 1998 and subsequent hiatus in funding. In the extension period we intend completing Task 4 of the statement of work; Immunohistochemical study of MMP family in surgical specimens from 30 breast cancer patients with microcalcifications and 30 controls.

Body

The first two specific aims of the proposal as outlined in the technical objective have been addressed, namely 1) study of the mitogenic response of cells in culture to HA

crystals, and 2) investigation and characterisation the induction of MMPs by HA crystals in cell culture. The results of which are summarised in the manuscript entitled 'Calcium Hydroxyapatite Promotes Mitogenesis and Induces Prostaglandin E_2 and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines'(attached in the appendix).

The third technical objective involved analysing the pattern of HA-associated MMPs in surgical specimens. This analysis is currently being initiated.

Additional data generated in the past year:

1) Anti-angiogenic property of calcium hydroxyapatite

Angiogenesis is the multistep process whereby new blood vessels develop from pre-existing vasculature, which plays a key role in the growth of solid tumours. In the model used, human endothelial cells are co-cultured with other human cells in a specifically defined medium. The endothelial cells initially form small islands within the culture matrix. They subsequently begin to proliferate and enter a migratory phase during which they move through the matrix to form threadlike tubule structures. These gradually join up (by 12-14 days) to form a network of anastomosing tubules which closely resembles the capillary bed found in the CAM assay. The tubules were visualised by staining for ICAM-2.

We found that control, unstimulated cultures formed a network of tubules following 12 days (Figure 1a). In contrast, following 12 days incubation with 18μg/cm^2 HA the endothelial cells remained in small islands in the matrix and no tubule formation was seen, demonstrating the anti-angiogenic property of HA (Figure 1b). The precise mechanism of this anti-angiogenic property is unknown.. However, we have previously shown that HA causes increased expression of several members of the MMP family including MMP-9 and -7. *In vivo*, endothelial synthesis of MMPs seems to have opposite effects on tumour angiogenesis, on the one hand facilitating extracellular matrix degradation and new blood vessel formation (Hiraoka, Allen et al. 1998) and on the other hand blocking angiogenesis by producing inhibitors of endothelial cell growth, including angiostatin (Kleiner and Stetler-Stevenson 1999).

2. HA causes upregulation of PGE_2 by induction of COX-1 and COX-2 in human fibroblasts

PGE_2 is an important mediator of joint degeneration in a variety of arthropathies. The cyclooxygenases (COX), of which there are two isozymes (COX-1 and COX-2), are responsible for PG formation. COX-2 is inducible by a variety of cytokines and mitogens and recent studies have also shown COX-1 to be inducible by certain agonists including lipopolysaccharide and vascular endothelial growth factor. To further elucidate the mechanism of HA crystal-induced PG production, we hypothesised that HA crystals upregulate both COX-1 and COX-2 expression leading to increased PGE_2 production.

As outlined in the original proposal we used normal human foreskin fibroblasts (HFF) as a model for breast stroma. We studied the effect of HA crystals on COX mRNA and protein expression. PGE_2 levels were also quantified using a commercial ELISA (Figure 2). Quiescent HFF were treated for varying times with HA crystals (100 μg/ml), phorbol myristate acetate (PMA) (1 μM) or left untreated. Untreated HFF were found to express COX-1 only. COX-2 mRNA was induced 4 hours post stimulation with HA crystals and subsequent expression of COX-2 protein

was seen by Western blot (Figures 3, 4). This induction was shown to be dose dependent and doses of 6μg/cm^2 and greater caused induction of COX-2 (Figure 5). Analysis of mRNA for COX-1 showed upregulation of transcript peaking at 30 hours post stimulation (1.32 fold increase). This increase was also seen at the protein level (Figure 6). PGE_2 production measured 4 and 30 hours after treatment was higher in HA-treated cultures (3.12ng/ml, 4.074ng/ml respectively) compared with untreated HFF (2.03ng/ml, 2.468ng/ml).

These studies indicate that HA crystals induce PGE_2 production in HFF through induction of both COX-1 and COX-2. We intend continuing these studies by co-culturing HFF with breast cancer cell lines as a model for stromal interaction with neoplastic cells (Ito, Nakajima et al. 1995). Elevated levels of PGE_2 have been widely reported in many human breast cancers as well as experimental murine mammary tumour models (Schrey and Patel 1995). Several studies with murine mammary tumour cells indicate that PGE_2 may have a multifunctional role in controlling growth, metastasis, and the host immune response in breast cancer (Rolland, Martrin et al. 1980; Fulton and Heppner 1985). Furthermore high levels of PGE_2 are often associated with estrogen receptor-negative tumours that exhibit a high metastatic potential (Rolland, Martrin et al. 1980).

3. Effects of calcium HA on cytokines in HFF

We examined the effect of HA crystals on cytokines IL-1B and TNF-a by RT-PCR. We found that incubation of 18μg/cm^2 HA with HFF cells caused induction of IL-1B mRNA. IL-1B mRNA was induced after 4 hours incubation and was maximal at 8 hours (Figure 7). HA was found to have no effect on TNF-a mRNA expression (data not shown).

Key Research Accomplishments in the past year

- Calcium hydroxyapatite crystals were found to induce an anti-angiogenic effect in an *in vitro* angiogenesis assay.
- Calcium hydroxyapatite crystals cause increased production of prostaglandin E_2 by induction of both cyclooxygenase–1 and cyclooxygenase-2 in human fibroblasts.
- Calcium hydroxyapatite causes induction of the cytokine IL-1B mRNA in HFF.

Reportable Outcomes

- Abstract submitted to Sixth Annual Biomedical Research Symposium, St. Vincent's Hospital, Dublin, Ireland, November 1999. 'Novel pathogenic mechanisms in breast cancer; role of calcium hydroxyapatite crystals'; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM. Winner of 'Best Poster Presentation'.
- Abstract submitted to Irish Association for Cancer Research Annual Meeting, Galway, Ireland, April 2000. 'Biological effects of calcium hydroxyapatite in breast cancer cell lines'; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM, Irish Journal of Medical Science (in press).

- Abstract submitted to Royal College of Surgeons in Ireland Research Day, April 2000, 'Calcium hydroxyapatite in breast cancer'; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM.
- Abstract submitted to the DoD Era of Hope Breast Cancer Research Program Meeting, Atlanta, USA, June 2000, entitled 'Novel pathogenic mechanisms in breast cancer; role of calcium hydroxyapatite crystals'; McCarthy GM, Westfall, PR, Christopherson, PA, and Morgan, M.
- Maria Morgan PhD, a post-doctoral fellow was awarded a post-doctoral research fellowship from the Department of Defence Breast Cancer Research and Material Command's office of Congressionally Directed Medical Research Program for proposal entitled 'Molecular mechanisms of calcium hydroxyapatite crystal-induced mitogenesis in breast cancer' (BC990714), covering period: July 2000 – July 2003. The application was based on preliminary work supported by this award.
- Maria Morgan PhD, was also subsequently awarded an Irish Health Research Board post-doctoral research fellowship (PD-06/99) but did not accept it.
- Manuscript entitled 'Calcium Hydroxyapatite Promotes Mitogenesis and Induces Prostaglandin E_2 and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines', Maria P. Morgan, Pamela A. Christopherson, Pamela R. Westfall, Lynn M. Matrisian and Geraldine M. McCarthy, is to be submitted to Advances in brief, Cancer Research. See appendix for copy of manuscript.
- Geraldine McCarthy hosted a mini-symposium on 'Synchrotron Radiation and Calcification in Breast Cancer', April 2000, sponsored by Bristol-Myers Squibb. Speakers included Dr. Rob Lewis Daresbury Synchrotron Laboratory, Warrington, UK, Dr. Keith Rodgers, Reader in Crystallography, Cranfield University, Wiltshire, UK, and numerous pathologists from both the UK and Ireland.
- Geraldine McCarthy (Principle Investigator) was awarded a grant from The Wellcome Trust for a project entitled 'Biological effects of calcium-containing crystals', covering period August 2000-August 2003. The application was based on preliminary work supported by this award.
- Proposal submitted to Bristol-Myers Squibb Foundation, Better Health for Women Foundation initiative in July 2000 based on results generated with the support of this award.

Conclusions

For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. The induction of MMPs in proliferating tissues could facilitate the clearing of basement membrane and/or connective tissue matrix components to make room for the multiplying cells as they expand. MMP induction and increased PGE_2 synthesis may be part of a programme of gene expression designed for malignant growth. These findings support our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and underlie the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

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APPENDICES

Includes figures 1-7 and manuscript

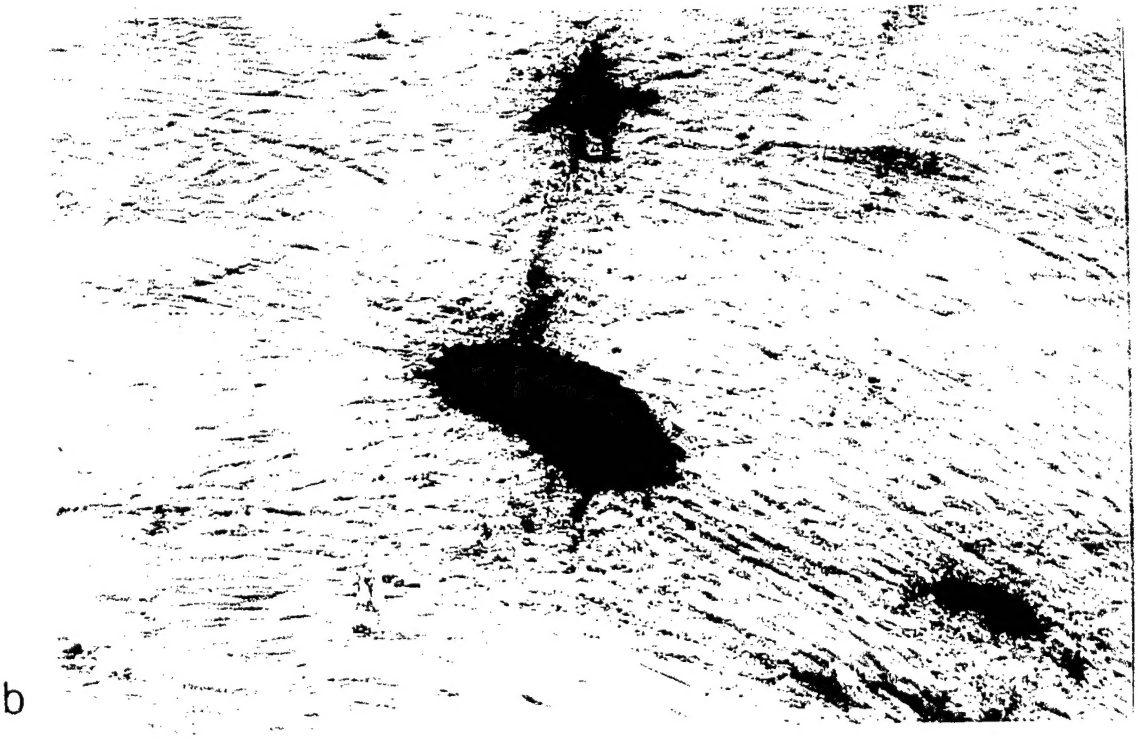

Figure 1. Effect of calcium hydroxyapatite on angiogenesis performed over 12 days. (a) Untreated cultures, showing tubule formation, (b) calcium hydroxyapatite added at 18μg/cm^2, no tubule formation.

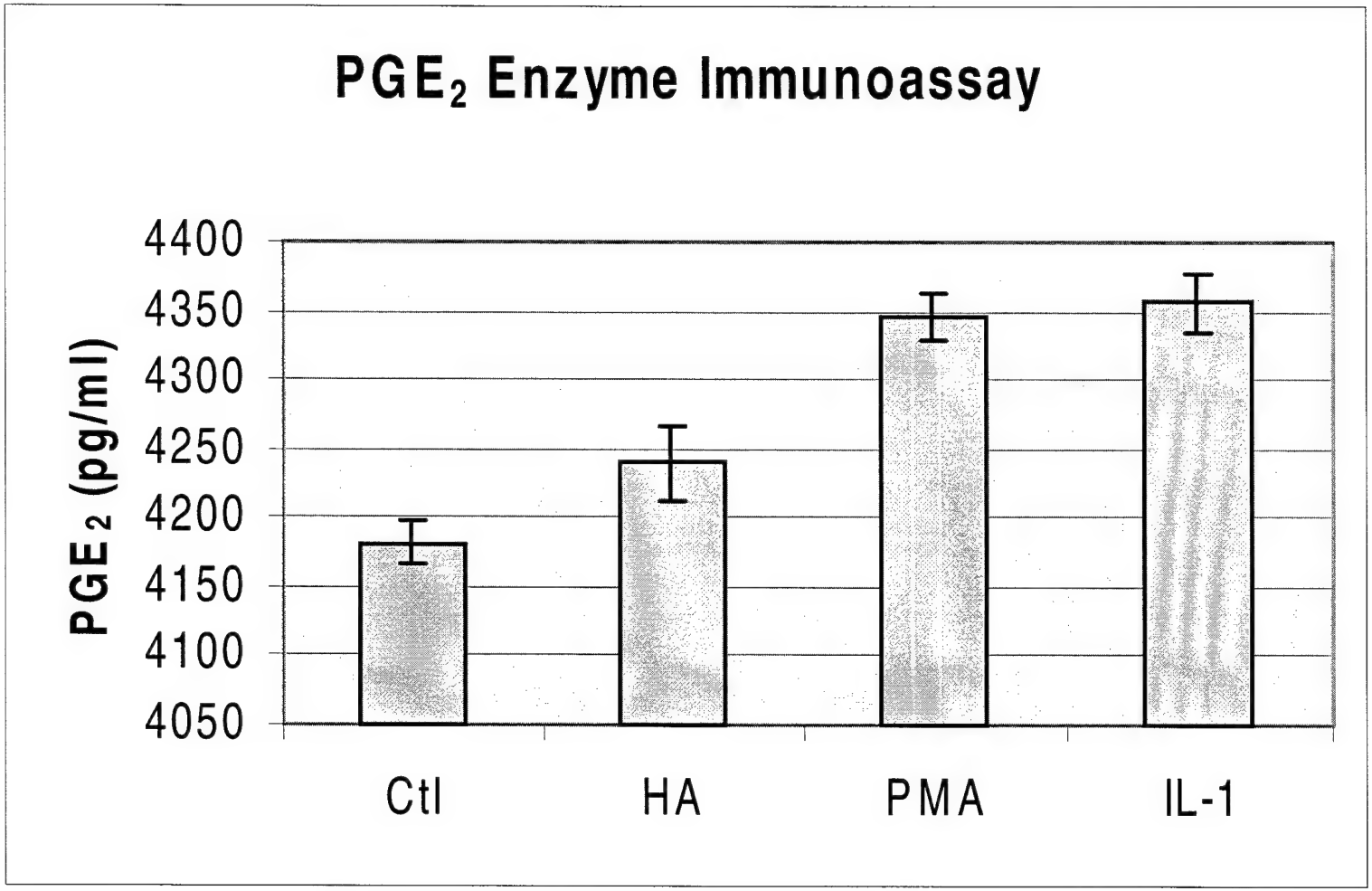

Figure 2. PGE_2 levels were measured by EIA. HA crystals caused an increase in PGE_2 production by HFFs.

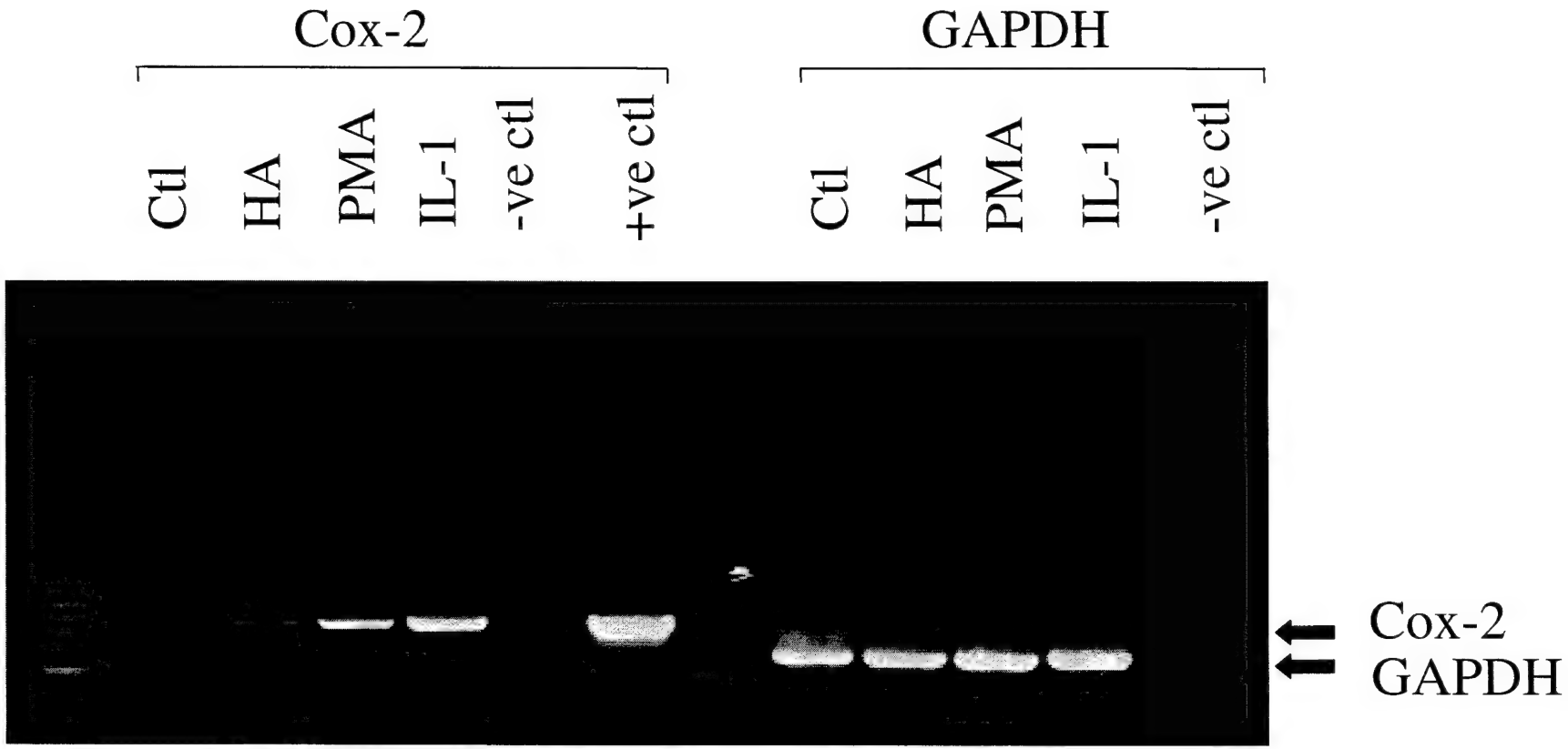

Figure 3. RT-PCR results showing induction of Cox-2 mRNA by HA crystals. Primers specific for Cox-2 and the constitutively expressed gene GAPDH were used.

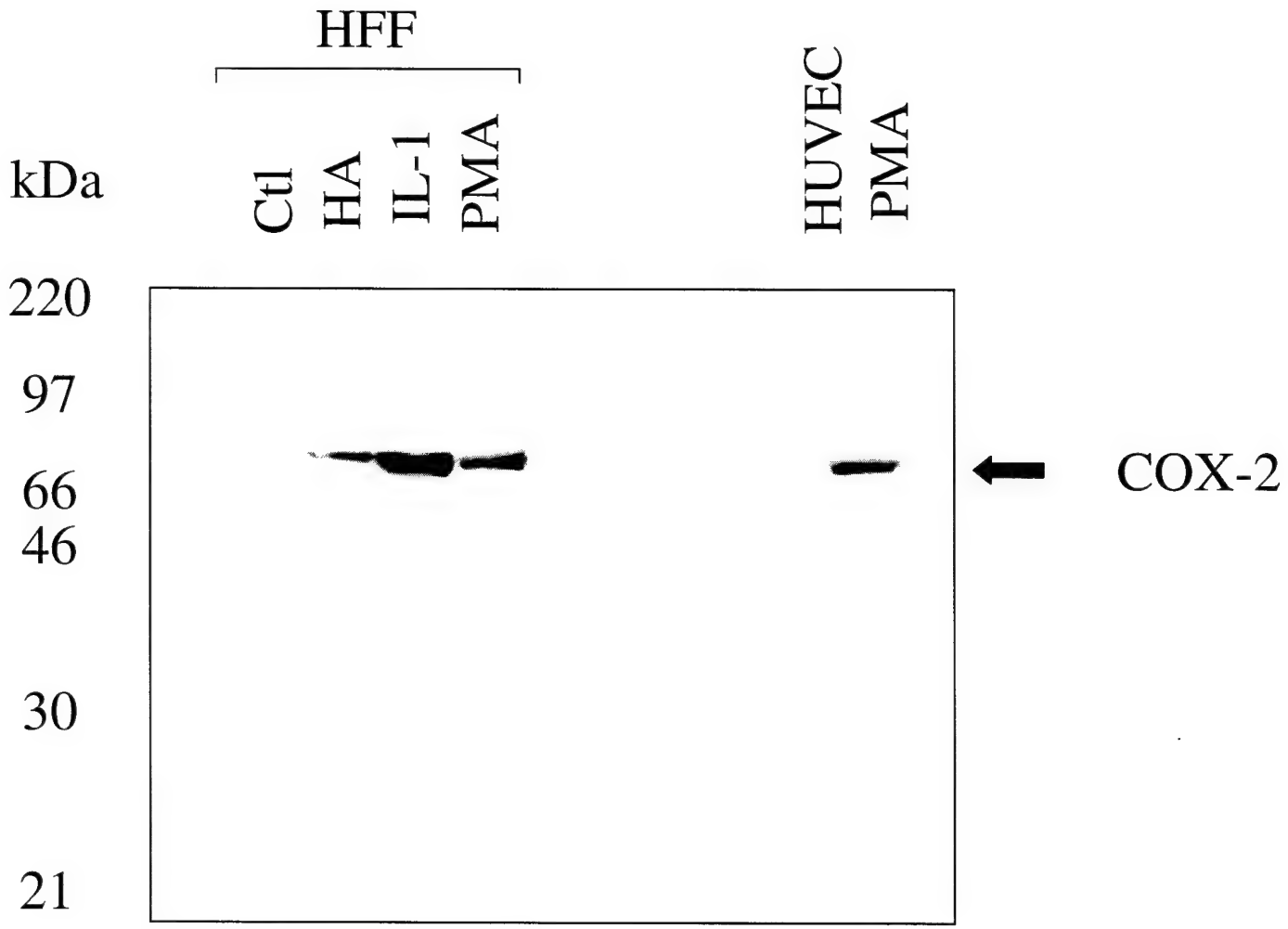

Figure 4. Western blot showing induction of COX-2 protein by HA crystals in HFF cells. COX-2 was also induced by IL-1 and PMA treatment. Positive control for COX-2 was PMA treated HUVEC cells.

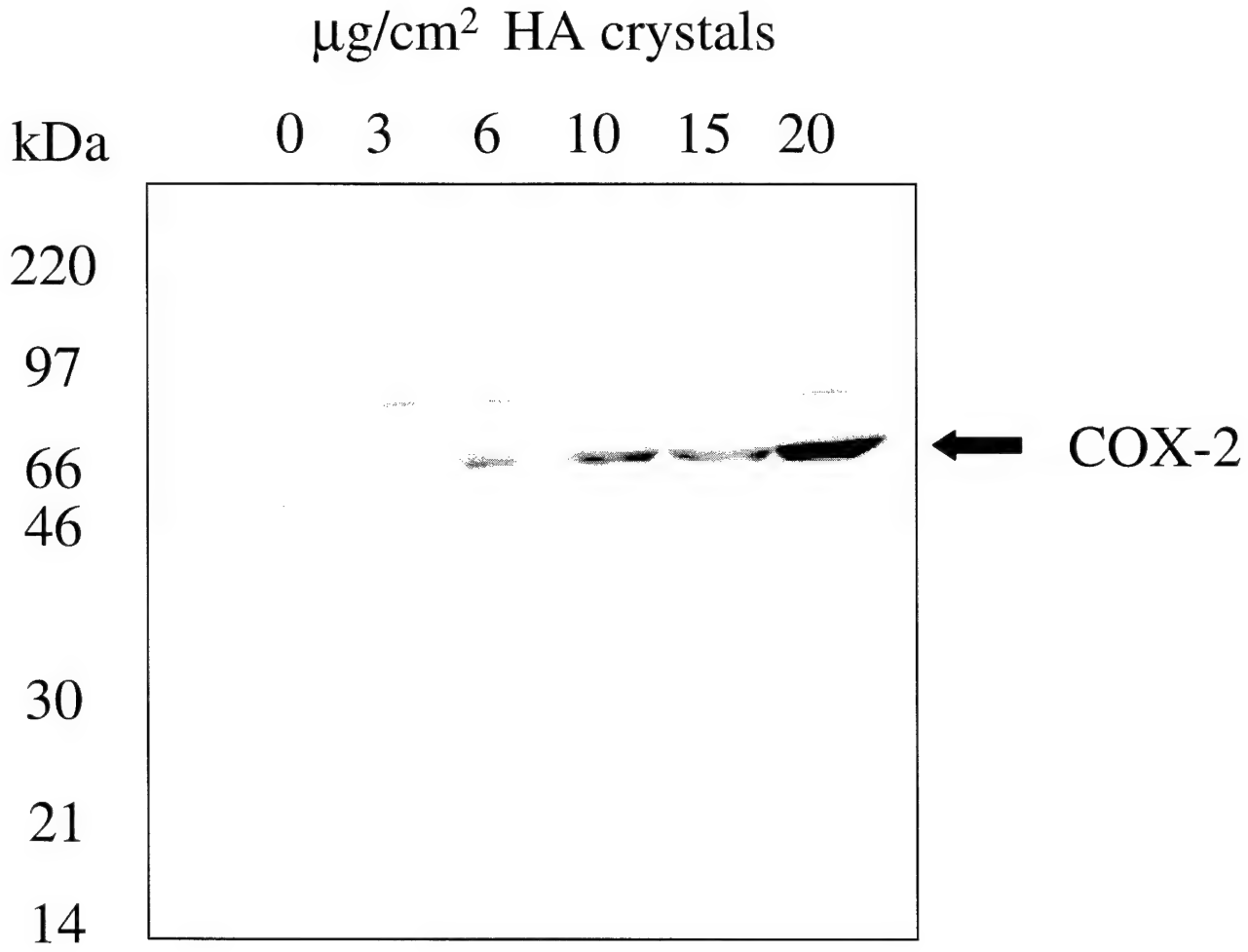

Figure 5. Western blot showing the HA crystal dose-related induction of COX-2 protein in HFF cells. Doses of 6μg/cm² and greater caused induction of COX-2.

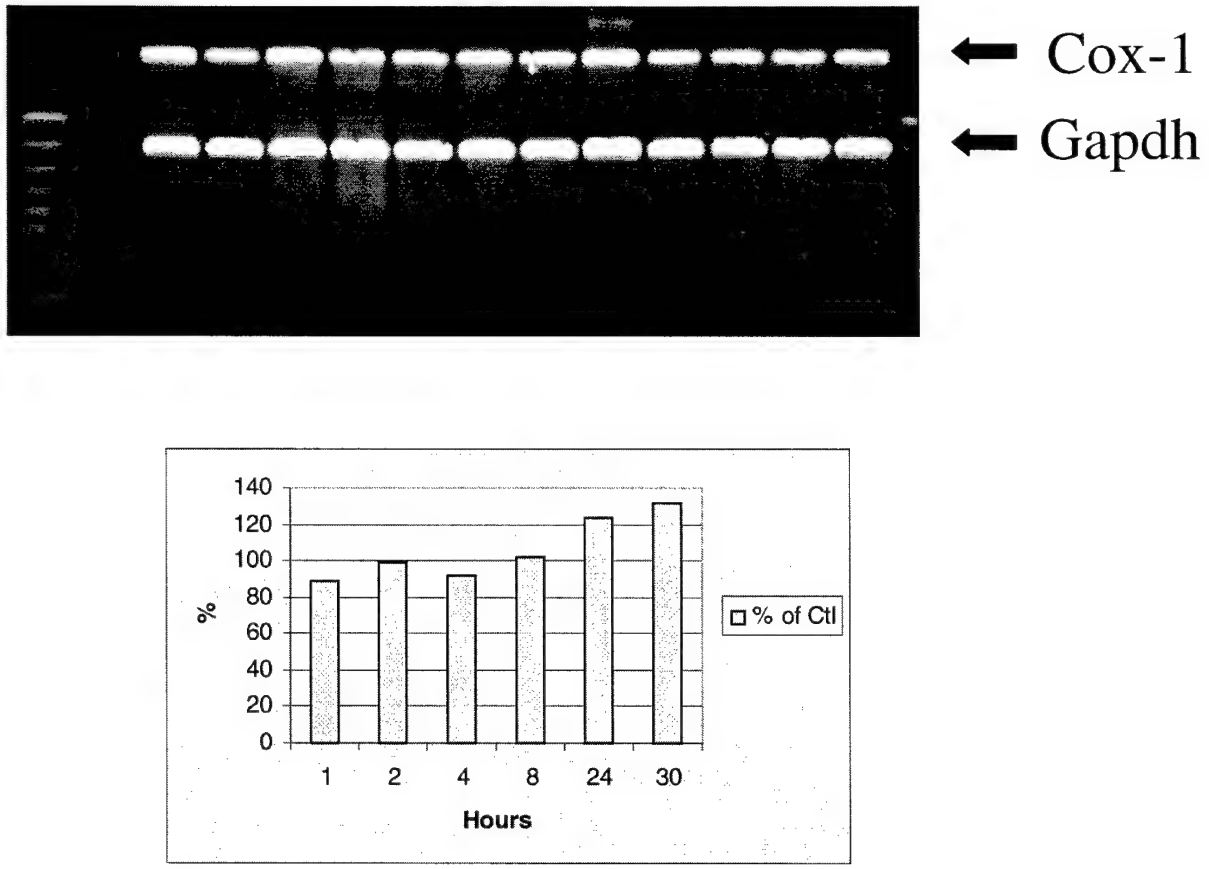

Figure 6. Time course showing upregulation of COX-1 mRNA by HA crystals. Densitometry was performed and values normalised for constitutively expressed GAPDH (shown in graph).

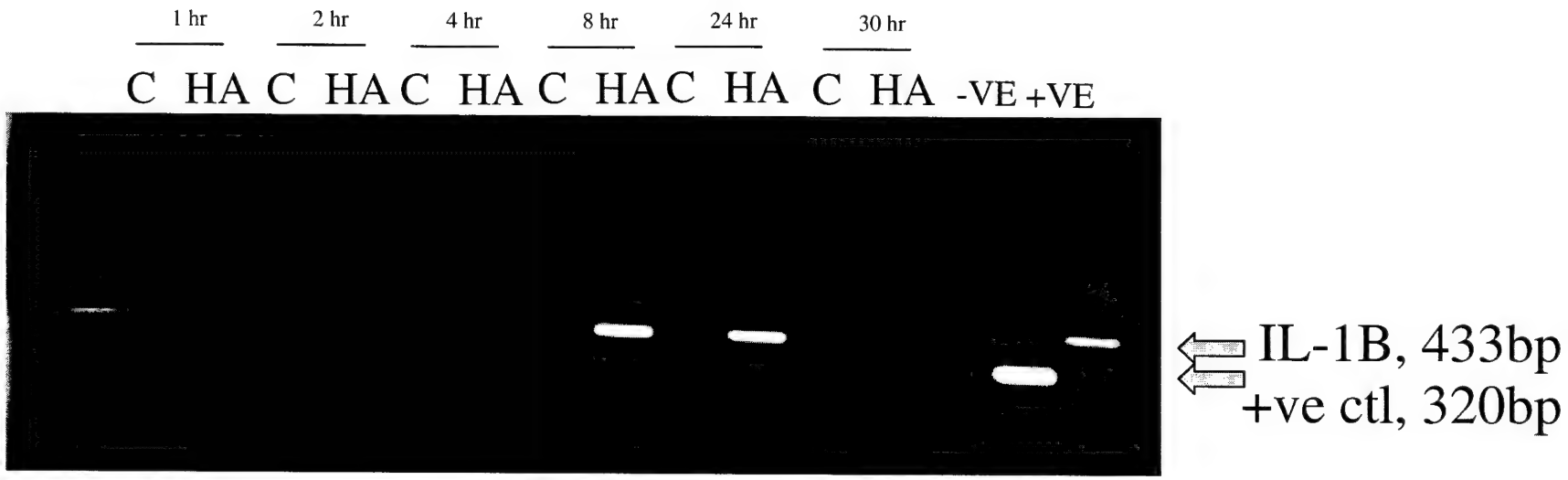

Figure 7. Time course of HA crystal induction of IL-1B mRNA in HFF by RT-PCR. IL-1B PCR product 433bp and plasmid +ve control 320bp

Calcium Hydroxyapatite Promotes Mitogenesis and Induces Prostaglandin E_2 and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines[1]

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Running Title: Biological Effects of Calcium Hydroxyapatite Crystals in Breast Cancer.

Keywords: hydroxyapatite, mitogenesis, matrix metalloproteinases, breast cancer, microcalcification.

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The abbreviations used are: HA, hydroxyapatite; MMP, matrix metalloproteinase; HMEC, human mammary epithelial cells; BM, basement membrane; ECM, extracellular matrix; PG, prostaglandin; EGF, epidermal growth factor; FBS, fetal bovine serum.

ABSTRACT

Radiographic mammary microcalcifications are one of the most pertinent diagnostic markers of breast cancer. Breast tissue calcification in the form of calcium hydroxyapatite (HA) is strongly associated with malignant disease. We tested the hypothesis that calcium HA may exert biological effects on surrounding cells, thereby facilitating breast cancer progression by amplifying the pathological process. Our findings show that HA crystals enhance mitogenesis in breast cancer cell lines MCF-7 and Hs578T, and also in normal human mammary epithelial cells (HMEC). HA crystals were also found to upregulate the production of a variety of matrix metalloproteinases (MMP), including MMP-2,-9 and –13 in MCF-7 and MMP–9 in HMEC cell lines. Finally, HA crystals were found to greatly augment prostaglandin E_2 levels in Hs578T cells. These results suggest that calcium HA crystals may play an active role in amplifying the pathological process involved in breast cancer.

INTRODUCTION

Radiographic mammary microcalcifications constitute one of the most pertinent markers of both benign and malignant lesions of the breast. Analysis of these microcalcifications by electron microscopy, microprobe analysis and X-ray diffraction has shown that in breast tissue two principle types of calcifications can be distinguished according to their structure and chemical composition [1]. Type I microcalcifications are composed of calcium oxalate in the form of weddellite crystals and type II microcalcifications consist of calcium phosphates in the crystalline form of hydroxyapatite (HA), ($Ca_{10}(PO_4)_5(OH)_2$), which is also the basic calcium phosphate found in mature bones and teeth. There is evidence that calcium phosphate and oxalate tend to be associated with different kinds of breast lesions [2]. The presence of oxalate-type microcalcifications appears to be a reliable criterion in favour of the benign nature of the lesion or, at most, of an *in situ* lobular carcinoma and are rarely associated with malignancy [3, 4]. In contrast, the calcifications associated with malignant breast lesions are generally formed by hydroxyapatite [5].

Although their diagnostic value is of great importance radiographically, the genesis of breast calcifications is unclear. The mineralization of breast tissue occurs by deposition of carbonated hydroxyapatite crystals in an extracellular matrix consisting of type I collagen and a variety of noncollagenous proteins. Among these, expression of the bone matrix proteins (bone sialoprotein, osteonectin, osteopontin) and also parathyroid hormone-related protein are believed to play an important role in the initiation and regulation of the deposition of microcalcifications [6]. The luminal calcifications associated with breast lesions appear to be the consequence of an active secretory process by the tumour cells and not solely the result of mineralisation of cellular debris and degenerate tumour cells [7]. The occurrence of microcalcifications

has not been shown to be significantly associated with age or primary tumour size. However, several studies have shown that survival of patients with mammographic microcalcification was significantly shorter than those without [8],[9]. A recent study by Tabar *et al.* also showed that the relative hazard of death from breast cancer was five times higher for tumours with casting-type calcifications than that for circular lesions with no calcifications [10].

The potent biological effects of calcium HA crystals are well recognised in other diseases unrelated to the breast. For example, crystals of basic calcium phosphate (BCP, term used to describe a mixture of predominantly HA, with small amounts of octacalcium phosphate and tricalcium phosphate) are common in osteoarthritic knee effusions. These crystals clearly potentiate joint damage as their presence and concentration correlates strongly with radiographic evidence and degree of cartilage degeneration [11]. The biological effects of HA crystals which promote articular damage have been well described, and include the induction of synoviocyte mitogenesis, accompanied by upregulation of several members of the MMP family leading to marked synovial proliferation and severe cartilage degeneration [12]. These properties may also be relevant in breast oncology.

There have been numerous histological ultrastructure studies of HA deposits in breast carcinomas. However, despite their potent biological effects in other systems and their association with poorer survival in breast cancer patients, to date there have been no investigations of their potential role in the growth and progression of breast tumours. In the present study, we investigated the pathogenic potential of calcium HA crystals in human breast cancer cell lines by studying their ability to induce mitogensis, and upregulate PGE_2 and MMP production.

MATERIALS AND METHODS

Crystal Synthesis and Preparation: HA crystals were synthesised by alkaline hydrolysis of brushite as previously described. Mineral prepared by this method has a calcium/phosphate molar ratio of 1.59 and contains predominately calcium hydroxyapatite as shown by Fourier transform infrared spectroscopy. The crystals were sterilised and rendered pyrogen-free by heating at 200°C for 90 min. Crystals were weighed and resuspended by sonication in Dulbecco's modified Eagles medium (DMEM).

Cell Culture: MCF-7 and Hs578T (American Type Culture Collection, Rockville, MD) were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 100mM sodium pyruvate and bovine insulin (4mg/ml). HMEC cells and mammary epithelial cell growth media were purchased from Clonetics, Biowhittaker, UK. All cell lines were maintained in a humidified incubator at 37°C with 5% carbon dioxide/95% air.

Stimulation of cells in culture: Cells were cultured to confluence and rendered quiescent by incubation in 0.5% FBS for 24 hr. Fresh 0.5% FBS containing media was then added and the cells treated with HA crystals (18μg/cm^2), EGF (0.1ng/ml), FBS (10%), or left untreated for 48 hr. Conditioned media and cell lysates were then collected for analysis.

[^{3}H]Thymidine Incorporation Assays: Cells were grown to confluence in 24-well plates and rendered quiescent by incubation in 0.5% FBS for 24 hr. [^{3}H]Thymidine (1μCi/ml) was added to the wells 23 hr after the addition of HA crystals (18μg/cm^2), EGF (0.1ng/ml), FBS (10%), or left untreated and pulse labelled for 1 hr. The cells were then washed and macromolecules were precipitated with 5% trichloroacetic acid

solution. Levels of trichloroacetic acid-precipitable ^{3}H were determined in quadruplicate, using a liquid scintillation counter (Wallac 1214 Rackbeta).

Gelatin Zymography: SDS-PAGE gels were prepared with gelatin (1mg/ml) co-polymerized in the 10% resolving gel and samples were separated under nondenaturing conditions. Following electrophoresis the gels were washed in 2.5% Triton X-100 for 30 min to remove the SDS and allow the MMPs to renature and then incubated for 24 hr at 37°C in 50mM Tris buffer, pH 7.4, containing 0.15M NaCl and 30mM $CaCl_2$. Gels were stained with Coomassie R-250 and destained with water.

Western Blots: Following electrophoresis of 10% SDS-PAGE gels, proteins were electrophoretically transferred to nitrocellulose membranes for 2 hours. Membranes were blocked in 2.5% non-fat dry milk. The membranes were then incubated for 3 hr with 1:500 dilution of primary anti-MMP-13 antibody (R4356, a gift from Peter Mitchell, Pfizer-Central Research Division, Groton, CT 06340). Secondary peroxidase-conjugated anti-rabbit IgG was used at a dilution of 1/5000. Immunoreactive bands were detected using enhanced chemiluminescence reagents (ECL-plus) (Amersham Pharmacia Biotech).

Prostaglandin E_2 Immunoassay: Cells were incubated in hanks hepes buffer with 50μM arachidonic acid for 15 minutes and samples collected. PGE_2 synthesis was measured using a commercially available PGE_2 immunoassay from R&D Systems, Oxon, UK.

Statistics: Statistical analysis was performed using the Wilcoxon Rank Sum test.

RESULTS

Calcium HA Enhances Mitogenesis in Human Breast Cancer Cell Lines.

We examined the mitogenic effect of treating both malignant and normal mammary cells with HA crystals *in vitro*. We also looked at the effect of EGF and FBS on the

cells. Mitogenesis was assessed by [^{3}H]thymidine incorporation assays. In all cell lines the addition of 18μg/cm^2 HA crystals for 24 hours enhanced mitogenesis above untreated cells. This increase was statistically significant for all cell lines ($p \leq 0.05$) (Figure 1a). The mean count per minute ± SD for unstimulated control cultures was; HMEC 455 ± 63; MCF-7 1823 ± 266; and Hs578T 3858 ± 535 compared to HA treated cultures; HMEC 568 ± 28; MCF-7 2610 ± 486; and Hs578T 7798 ± 558. The mitogenic effect of HA crystals was not quite as potent as EGF; HMEC 750 ± 25; MCF-7 2890 ± 433; and Hs578T 8728 ± 1022. The cell lines were routinely grown in 10% FBS. We also performed cell counts using a haemocytometer to confirm that the increased DNA synthesis was accompanied by an increase in cell number. The mitogenic effect shown by increased thymidine incorporation was confirmed by a statistically significant increase in cell numbers 48 hours following stimulation ($p \leq 0.05$) for all cell lines (Figure 1b). The mean ± SD($x10^5$) number of cells in unstimulated control cultures for each cell line was; HMEC 1.1 ± 0.153; MCF-7 2.0 ± 0.35; and Hs578T 1.85 ± 0.59. The number of cells in cultures incubated with HA was increased above unstimulated cultures; HMEC 2.05 ± 0.451; MCF-7 3.1 ± 0.216; and Hs578T 3.6 ± 0.48. The mean number of cells in cultures treated with EGF was; HMEC 3.1 ± 0.768; MCF-7 5.8 ± 0.141; and Hs578T 4.0 ± 1.05.

Calcium HA Upregulates Matrix Metalloproteinase Production in Human Breast Cancer Cell Lines.

In this study we investigated the effect of HA crystals on MMP-2 and MMP-9 expression using gelatin zymography and MMP-13 expression by Western blotting. 18μg/cm^2 HA crystals caused upregulation of MMP-2 and MMP-9 activity in MCF-7 cells following 48hr stimulation. Figure 2a shows a zymogram of MCF-7 conditioned

media with bands of lytic activity at 92 and 72 kDa representing MMP-9 and –2 respectively. Gelatin zymography also showed upregulation of MMP-9 activity in HMEC cells in response to HA crystals (Figure 2b). In contrast HA stimulation had no effect on MMP-2 or –9 expression in Hs578T cells (data not shown). MMP-13 protein production was also found to be upregulated in MCF-7s when treated with HA crystals (Figure 2c).

Effect of Calcium HA on Prostaglandin E_2 Synthesis in Human Breast Cancer Cell Lines.

We examined the effect of HA crystals on PGE_2 production in breast cancer cell lines. We found that the biologically aggressive, invasive Hs578T cell line possessed a higher constitutive level of PGE_2 (mean pg/ml ± SD) than the MCF-7 cells (Hs578T 204 ± 62; MCF-7 53 ± 7). In addition, HA crystals were found to further augment PGE_2 production in Hs578T cells (1608 ± 220) but a similar upregulation was not seen in MCF-7 cells (78 ± 9) (Figure 3). Treatment with PMA and IL-1 also caused increased production of PGE_2 production in Hs578T cells (4733 ± 475, 3998 ± 135 respectively).

DISCUSSION

The biological effects of calcium HA which may promote breast cancer were investigated *in vitro* in our laboratory and properties of calcium HA have been observed which emphasise its pathogenic potential. The first is its ability to promote mitogenesis, possibly amplifying the malignant process by leading to aggravation of tumour growth. In this study we report that HA increases mitogenesis in both normal and malignant mammary cell lines. We have previously shown that HA crystals

stimulate mitogenesis of quiescent cultured human foreskin fibroblasts and adult articular chondrocytes in a concentration dependent fashion [13]. The mechanism of HA crystal-induced mitogenesis in human foreskin fibroblasts involves two processes; (1) a fast membrane associated event involving protein kinase C and MAP kinase activation, nuclear factor-κB induction and expression of proto-oncogenes *c-fos* and *c-myc* and; (2) the relatively slow endocytosis and intracellular dissolution of the HA crystals raising intracellular calcium causing the activation of a number of calcium-dependent processes leading to cell proliferation [13]. The precise molecular mechanism of HA induced-activation of mammary cells is currently being investigated.

The early proliferative stages of breast cancer are characterised by a continuous basement membrane (BM) separating the hyperplastic epithelial cells from the surrounding stroma. Pathologically, the transition from *in situ* to invasive carcinoma is usually accompanied by disorganisation and interruption of the BM caused by an enhanced process of proteolysis contributing to the escape of breast cancer cells into neighbouring tissues, eventually leading to the formation of distant metastases. MMPs are members of a unique family of proteolytic enzymes that can degrade native collagens and other ECM components [14]. Previous experimental and clinopathological studies have shown good correlations between expression of MMPs and the invasive phenotype of tumour cells [15]. The inducability of the MMPs by a diverse range of extracellular stimuli has been well documented, including growth factors, phorbal esters, hormones, steroids, and adhesion molecules [14]. The differing effects of HA on MMP expression in the cell lines examined in this study may reflect their contrasting states of differentiation. MCF-7 have an epithelial-like phenotype, are estrogen receptor (ER) positive and are weakly invasive, while

Hs578T have a more fibroblast-like phenotype, are ER negative and are highly invasive. Studies have shown that HA crystals are potent inducers of MMP-1, –3 and –9 in human foreskin fibroblasts and synoviocytes. Cheung and co-workers have recently shown that HA crystals induce MMP-1 expression through an ERK 1 and 2 MAPK pathway also involving c-fos/AP-1 and RAS signalling pathways [16].

Elevated levels of PGE_2 have been widely reported in many human breast cancers as well as experimental murine mammary tumour models [17]. Several studies with murine mammary tumour cells indicate that PGE_2 may have a multifunctional role in controlling growth, metastasis, and the host immune response in breast cancer [18]. Furthermore high levels of PGE_2 are often associated with estrogen receptor-negative tumours that exhibit a high metastatic potential [18]. Calcium-containing crystals have previously been reported to stimulate PG release from cultured mammalian cells accompanied with the release of proteases. In the present study the differential regulation of PGE_2 in cell lines MCF-7 and Hs578T may be caused by upstream regulation of cyclooxygenase (COX) expression. Differential expression and regulation of COX-1 and –2 has been reported in two human breast cancer cell lines (MDA-MB-231 and MCF-7) where COX-2 expression and induction was reported to be influenced by hormone status and metastatic phenotype [19]. Furthermore, we have also recently shown that HA crystals cause induction of COX–2 mRNA and protein in human fibroblasts [20].

For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. The induction of MMPs in proliferating tissues could facilitate the clearing of basement membrane

and/or connective tissue matrix components to make room for the multiplying cells as they expand. MMP induction and increased PGE_2 synthesis may be part of a programme of gene expression designed for malignant growth. These findings support our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and emphasise the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

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Figure Legends

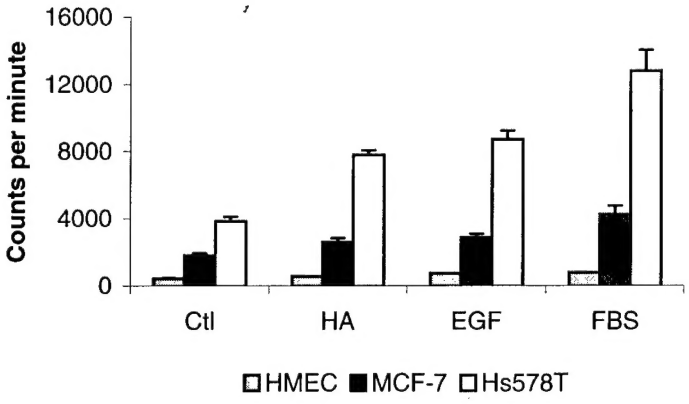
Fig. 1 Mitogenic effect of hydroxyapatite crystals on breast cancer cell lines. Confluent, quiescent cultures of HMEC, MCF-7 and Hs578T cells were stimulated with HA crystals (18μg/cm^2), EGF (0.1ng/ml), FBS (10%), or left untreated (Ctl). (a) After 24hr cells were pulse labelled with ^{3}H-thymidine (1μCi/ml) for 1 hr. Levels of trichloroacetic acid-precipitable ^{3}H were determined in quadruplicate, using a liquid scintillation counter. HA caused a statistically significant increase in ^{3}H-thymidine uptake over untreated cells ($p \leq 0.05$). (b) Cell counts were performed using a haemocytometer following 48hr stimulation. HA also caused a statistically significant increase in cell numbers ($p \leq 0.05$).

Fig. 2 Hydroxyapatite (HA) crystals induce gelatinase activity in human breast cancer cell lines MCF-7 (a) and HMEC (b). Confluent, quiescent cells were stimulated with HA crystals (18μg/cm^2), EGF (0.1ng/ml), FBS (10%), or left untreated (Ctl) for 48hr. Conditioned media was then collected and analysed by electrophoresis on a 10% polyacrylamide gel containing 1mg/ml gelatin. After overnight incubation at 37°C, gels were stained with Coomassie blue. Digestion of the gelatin substrate is seen as clear band.

Effect of HA crystals on MMP-13 protein expression in MCF-7 cells (c). CM was analysed by Western blot using a polyclonal antibody to MMP-13 (R4356).

Fig. 3 Effect of hydroxyapatite (HA) crystals on prostaglandin E_2 (PGE_2) synthesis in MCF-7 and Hs578T cells. Confluent, quiescent cells were stimulated with HA crystals (18μg/cm^2), phorbol myristate acetate (PMA) (1μM), IL-1 (2.5ng/ml), or left untreated (Ctl) for 8hr.

A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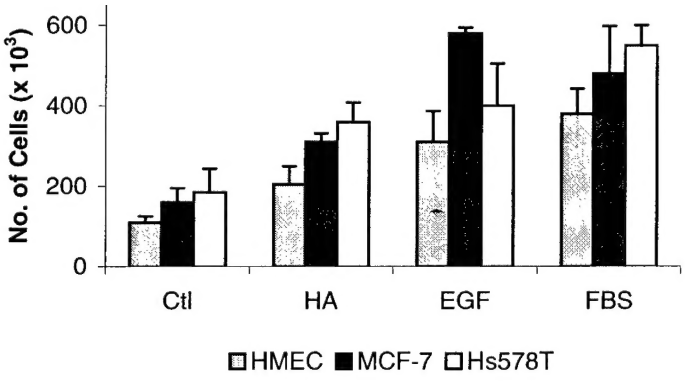
12000
8000
4000
0
Counts per minute
Ctl
HA
EGF
FBS
HMEC MCF-7 Hs578T

B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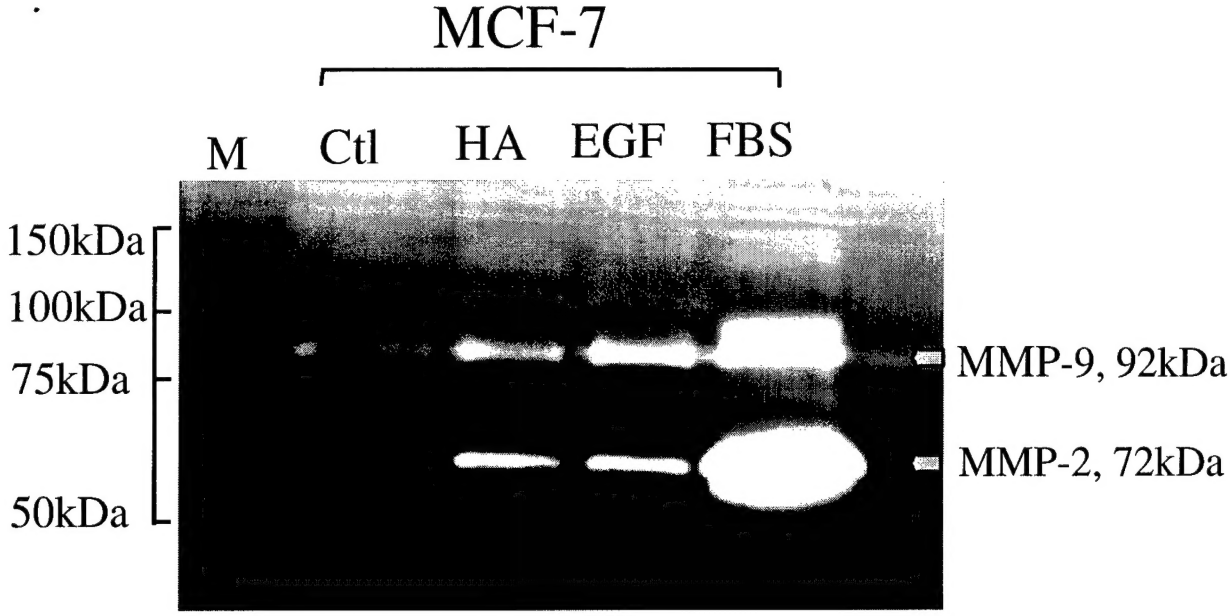
No. of Cells (x 10^3)
Ctl
HA
EGF
FBS
HMEC MCF-7 Hs578T

FIGURE 1

A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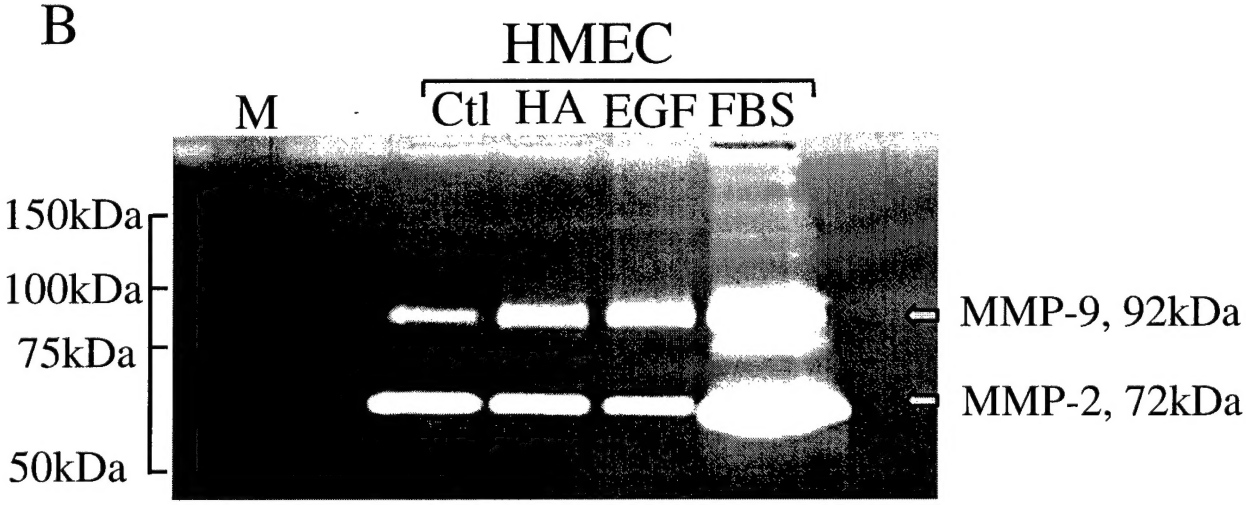
B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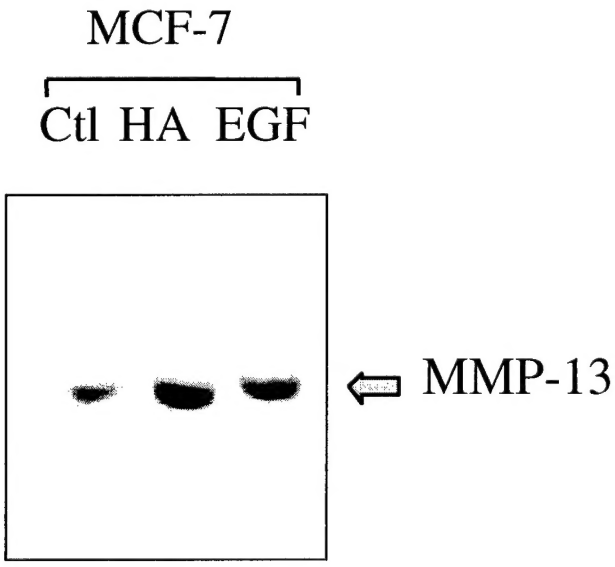
MMP-2, 72kDa

C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Ctl
HA
EGF
MMP-13

FIGURE 2

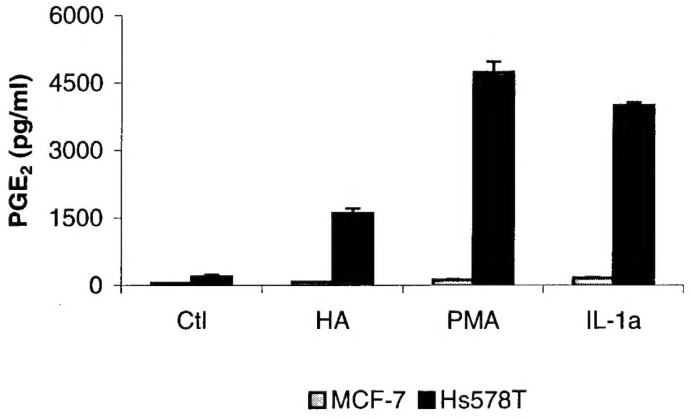

FIGURE 3